670. *The Action of Ionizing Radiations and of Radiomimetic Substances on Deoxyribonucleic Acid. Part II. The Effect of Oxygen on the Degradation of the Nucleic Acid by X-Rays.*

By J. A. V. Butler and B. E. Conway.

Degradation of the nucleic acid by *X*-rays, as shown by the decrease of viscosity, ceases when irradiation ceases provided that the solution has been prepared and irradiated under oxygen-free conditions. The after-effect is thus due to a product of molecular oxygen. The product is not hydrogen peroxide, since this cannot be detected in nucleic acid solutions irradiated in oxygen.

The effect of *X*-rays on solutions of deoxyribonucleic acid (calf thymus) has been studied by Sparrow and Rosenfeld (*Science*, **104**, 245, 1946), C. G. Butler (*Canadian J. Res.*, 1949, **27**, B, 972), Taylor, Greenstein, and Hollaender (*Biochem. Arch.*, 1948, **16**, 19; *Cold Spring Harbor Symp.*, 1947, **12**, 237), and by Scholes, Stein, and Weiss (*Nature*, 1949, **164**, 709). The first group of authors found that increasing doses of *X*-rays caused an increasing degradation of the nucleic acid, as shown by (*a*) loss of viscosity, especially the abnormally high viscosity observed at low rates of shear, (*b*) loss of streaming birefringence, and (*c*) changes in the sedimentation diagram which suggested the breaking up of the nucleic acid into smaller particles. They also observed that the decrease of viscosity continued after irradiation ceased. The later authors observed liberation of ammonia and other chemical changes which they considered to be due to breakdown of the purine bases. The *X*-ray doses used were, however, greatly in excess of those required to produce almost complete loss of the characteristic viscosity of the nucleic acid.

We have been interested in the " after-effect " of *X*-rays on account of its possible relation with the changes of viscosity produced by compounds of the " mustard " class (preceding paper, *Nature*, 1950, **165**, 714). Taylor *et al.* rejected the possibility that this was caused by hydrogen peroxide formed during irradiation when they found that between 10^{-2} and 10^{-5}M. it had no appreciable effect on the viscosity of the aqueous solutions of the thymus nucleic acid.

We have found that the after-effect does not occur in solutions prepared in the absence of oxygen. Identical solutions of nucleic acid in water, prepared in the absence of oxygen and subsequently (*a*) saturated with air or oxygen and (*b*) maintained in an oxygen-free atmosphere, were exposed to *X*-rays side by side in similar tubes. Viscosities were determined (in air) after dilution to 0·1% (see Fig. 1). In the absence of oxygen there is a small change of viscosity in the first ½—1 hour after the end of the irradiation, but the viscosity then remains quite constant for many hours, while that of the solution irradiated in oxygen continues to decrease. This shows that most of the after-effect of irradiation is connected with the presence of molecular oxygen in the solutions.

Since hydrogen peroxide had a marked degrading effect on our preparations of thymonucleic acid, at concentrations of the order of 10^{-3}M., and since (Fricke, *J. Chem. Physics*, 1934, **2**, 556; Allen, *J. Phys. Coll. Chem.*, 1948, **52**, 479) it is formed by *X*-irradiation of solutions of oxygen in pure water, by reactions such as

$$H + O_2 \longrightarrow HO_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1a)$$

$$2HO_2 \longrightarrow H_2O_2 + O_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1b)$$

the possibility existed that in our preparation the after-effect was due, or partly due, to hydrogen peroxide. We were, however, unable to detect this substance in solutions of nucleic acid

saturated with oxygen and exposed to 20,000 r., either by the liberation of iodine from potassium iodide, or by means of the pertitanic acid reaction (Allen, *Ind. Eng. Chem., Anal.*, 1945, **17**, 425). These tests gave unequivocal positive results for 10^{-5}M-hydrogen peroxide in nucleic acid solutions. Since hydrogen peroxide in a concentration of the order of 10^{-3}M. would be required to produce the observed changes of viscosity, we conclude that it is not the active agent.

Experimental.

The thymus nucleic acid was prepared as described in Part I (preceding paper). The deoxygenated solutions were prepared as follows. The nucleic acid was weighed into the vessel *A* (Fig. 2), which was then attached to the nitrogen-purification train. The vessel and the ampoules attached to it were evacuated and filled with nitrogen, twice, and the ampoules were finally left evacuated. Twice distilled water, boiled out in *B* and saturated with nitrogen, was then forced over into *A* by nitrogen pressure to give the volume required for a 0·2% solution. The nitrogen used was deoxygenated by passage through a furnace *C* at 500° containing copper filings. (It is estimated that the oxygen content of the solutions was less than 10^{-6} mol./l.) Nitrogen was bubbled through the mixture until complete dissolution of the

Fig. 1.

Change of viscosity, with time, of 0·1% thymus nucleic acid solution after termination of irradiation in presence and absence of oxygen.

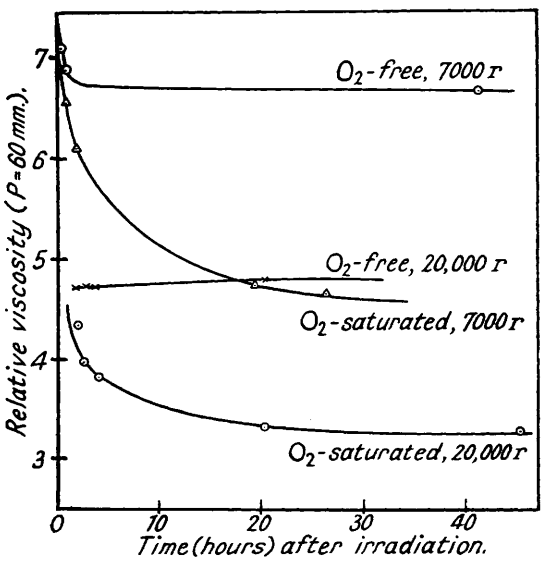
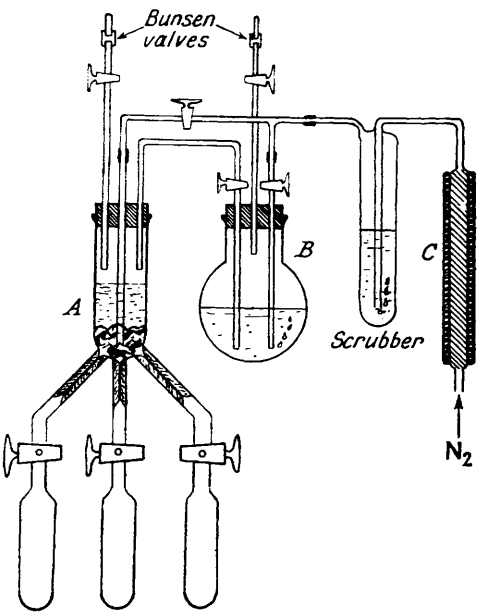


Fig. 2.

Apparatus for preparation of deoxygenated solutions.



nucleic acid had occurred (2 days). Suitable amounts of the solution were then let into the evacuated ampoules. After the taps had been closed the ampoules were cut away from the vessel *A* and subjected to *X*-irradiation by a 400-kv. *X*-ray machine (half-value layer of radiation, 3·6 mm. Cu) at a dosage rate of 206 r./min. to dosages of 7000, 14,000, 20,000, and 40,000 r. The ampoules were then opened and their contents diluted to 0·1% and 0·05% with distilled water. The relative viscosities at various pressure differences were determined in Frampton viscometers. The sedimentation and diffusion coefficients of the material subjected to doses of 7000 and 40,000 r. are reported in Part III (following paper).

Fig. 3, which assembles results obtained with several samples of thymus nucleic acid, shows the effect of the *X*-ray doses on the viscosity at a concentration of 0·1% in aqueous solution. The observations are fairly consistent and show that 7000 r. causes a fairly large drop in viscosity and that with 40,000 r. the loss of viscosity is almost complete.

Tests for hydrogen peroxide and other peroxides in oxygen-irradiated nucleic acid solutions were made by : (1) potassium iodide solution (1 ml. ; 50%) in 2N-hydrochloric acid (25 ml.) containing traces of ammonium molybdate and preserved in nitrogen (two drops were added with starch to 2 ml. of nucleic acid solution) and (2) titanous chloride solution (1 ml. ; 15%), hydrochloric acid solution (20 ml. ; 10%), and water (70 ml.) decolorised with a little hydrogen peroxide (Allen, *loc. cit.*). These tests are sensitive to hydrogen peroxide and hydroperoxides to 10^{-7}—10^{-8}M. (Ubbelohde and Egerton, *Phil. Trans.*, 1935, **234**, *A*, 491), but give no reaction with dialkyl peroxides. Although there was no immediate liberation of iodine in test (1), some iodine was liberated when the solution of nucleic acid, irradiated in oxygen, was left with the reagent for 10 hours. Oxygen itself causes a slow liberation of iodine under these conditions,

but that produced by the oxygen-irradiated nucleic acid was greater than that liberated by an un-irradiated control or by a control which has had a similar amount of irradiation in nitrogen. When the oxygen-irradiated solution of nucleic acid was heated to 90° for 1 minute, the iodine liberated in 10 hours was considerably greater. No titanium colour appeared in test (2) after storage for the same time.

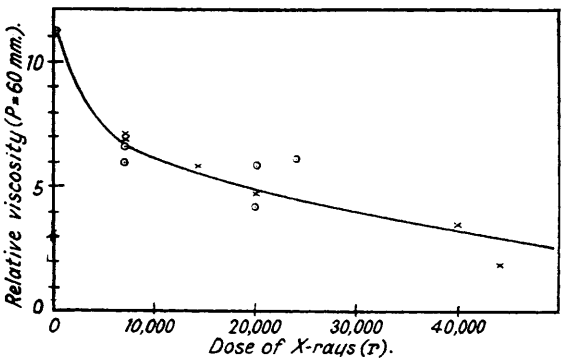
Observations were also made on simpler substances containing groups present in the nucleic acid. 1% Sodium dihydrogen phosphate, irradiated with 20,000 r. in the presence of oxygen, gave an immediate iodine liberation equivalent to $2{\cdot}5 \times 10^{-5}$ M-hydrogen peroxide. The colour developed in the titanium test also corresponded approximately to the same concentration.

A nucleotide (ammonium guanylate) similarly irradiated in 1% solution gave a very faint colour reaction with the titanium test and an immediate colour with hydriodic acid, corresponding to about 1×10^{-5}M-hydrogen peroxide.

Ferricyanide–ferric alum mixture (Ubbelohde and Egerton, *loc. cit.*), which is sensitive to 10^{-6}M-hydrogen peroxide and to 10^{-5}M-hydroperoxides, gave no reaction with the oxygen-irradiated (20,000 r.) nucleic acid. Ferrous thiocyanate was converted into the red ferric complex at a rate not markedly different from that of the control.

FIG. 3.

Effect of X-*rays on the viscosity of* 0·1% *thymus nucleic acid solutions, measured after irradiation.*



—○—○—○— *Irradiated in air or oxygen.*
—×—×—×— *Irradiated in nitrogen.*

Effect of Hydrogen Peroxide on Different Preparations of Nucleic Acid.—Although 10^{-3}M-hydrogen peroxide had a marked degrading effect on our own preparations of thymonucleic acid, a sample of the substance given us by Professor R. Signer was unaffected and was in that respect similar to that of Taylor, Hollaender, and Greenstein (*loc. cit.*). It has been shown by Butler and Smith (*Nature*, 1950, **165**, 847) that the action of hydrogen peroxide is greatly increased by ferrous ions. 10^{-5}M-Ferrous sulphate, when added to a solution of Signer's thymonucleic acid containing 5×10^{-3}M-hydrogen peroxide, produced a rapid fall of viscosity and it was appreciably active at 10^{-6}M. Corresponding concentrations of ferric salts had no effect. Since the concentration of ferrous iron is much smaller than that of hydrogen peroxide, there must thus be some mechanism, *e.g.*, a chain process, by which its action is continued. The difference between our preparations and those of Signer and of Taylor, Greenstein, and Hollaender could thus be accounted for by the presence, in ours, of a small quantity of ferrous or similarly-acting salts, probably stabilized by combination with the nucleic acid. This could easily have arisen, since the thymus glands were minced in a tinned mincer and also at one stage our crude preparation was centrifuged in a Sharples super-centrifuge with an iron rotor. Another difference in the method of preparation is that Signer, and Taylor, Greenstein, and Hollaender finally precipitated the nucleic acid with alcohol, while ours was freeze-dried. Freeze-drying a solution of Signer's material in water did not change its behaviour with respect to the effect of hydrogen peroxide. The absence of effect of hydrogen peroxide on Signer's and Taylor, Greenstein, and Hollaender's preparations could not therefore be ascribed to the presence of alcohol, unless this alcohol is very firmly held. Signer's preparation, however, showed a similar after-effect to ours, after irradiation with 7000 r. in the presence of oxygen. After dissolution of our product in water and re-precipitation with alcohol, the effect of hydrogen peroxide was much reduced, confirming that this effect was due to a removable substance.

DISCUSSION.

These experiments show that the after-effect is produced by the action of a primary product of the radiation with molecular oxygen. This might be, for example, the radical HO_2 formed in reaction 1*a*. Since the concentration of such radicals will be small, reaction 1*b*, giving hydrogen peroxide, cannot be expected to occur to any appreciable extent when other substances with which HO_2 can react are present. The radical HO_2 could, however, react with nucleic acid,

e.g., at the phosphoryl groups, forming a peroxidic derivative which undergoes a further slow change, resulting in the degradation of the nucleic acid.

We have very little information which would characterize the intermediate product. If nucleic acid is represented by X the simplest formulation of such a product is XO_2H, but it has been shown that the intermediate does not give the typical tests of a hydroperoxide and is more akin in its reactions to dialkyl peroxides. The degradation might be the result of an oxidative rearrangement, or possibly of the slow liberation of hydroxyl radicals, by $XO_2H \longrightarrow XO + OH$, which have been shown to be capable of degrading nucleic acid.

These results are of biological interest in view of the observations that the radio-sensitivity of plants and animals is enhanced by the presence of oxygen during the irradiation. Thus Thoday and Read (*Nature*, 1947, **160**, 608; 1949, **163**, 133) found that the presence of oxygen in the water was an important factor in the radio-sensitivity of the root-tips of *Vicia faba*. Hayden and Smith (*Genetics*, 1949, **34**, 26) found a reduced sensitivity of barley seeds when *X*-irradiated in a vacuum. Giles and Riley (*Proc. Nat. Acad. Sci.*, 1949, **35**, 640) have shown that the incidence of *X*-ray-induced chromosome rearrangements is reduced when air is replaced by nitrogen and increased in pure oxygen. Baker and Sgourakis (*ibid.*, 1950, **36**, 176) have obtained similar effects with *Drosophila* sperm.

Since various studies in enzymes (ribonuclease, Holmes, *Nature*, 1950, **165**, 266; carboxypeptidase, Dale, Gray, and Meredith, *Phil. Trans.*, 1949, **242**, *A*, 33) have shown no influence of oxygen on their radio-senstivity, it would appear that the nucleic acid behaviour described above provides a possible basis of the biological effects.

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